Antibody engineering

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Among the most important advances in antibody engineering of this past year is the advent of new tools to study the relationship between protein (including antibody) structure and function. Very rapid large-scale mutational analysis of antibodies is now possible by using *in vitro* transcription and translation. Ribosome display is a rapidly evolving technology for modifying antibody function that offers several potential advantages over phage display.

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Abbreviations

Ag	antigen
BsIgG	bispecific IgG
CDR	complementarity-determining region
FcRn	neonatal Fc receptor
FR	framework region
gIIIp	M13 gene III protein
PCR	polymerase chain reaction
scFv	single-chain variable region fragment
SIP	selectively infective phage
V_H	heavy chain variable domain
V_L	light chain variable domain

Introduction

In this review, we will focus on the important advances of 1997 and early 1998 in understanding the relationship between antibody structure and function, together with methods, such as *Escherichia coli* expression and new library technologies, that facilitate antibody engineering. We have minimized the overlap with other recent reviews on related topics: antibody engineering [1,2]; catalytic antibodies [3,4]; bivalent and bispecific antibody fragments [5]; diabodies [6]; antibody fragment expression in *E. coli* [7]; human antibodies from phage libraries [8,9] and transgenic mice [10]; intracellular antibodies [11]; selectively infective phage [12,13]; and immunotoxins [14].

The molecular basis of antigen–antibody interactions

Understanding the molecular mechanisms of antibody affinity and specificity towards a given antigen is a key goal in antibody engineering. For example, do a few or most of the antibody residues contacting the antigen contribute to the energetics of binding? Mutational analysis of an idiotope–anti-idiotope complex indicated that nearly all of the contact residues at the protein–protein interface play a demonstrable role in binding ($\Delta\Delta G > 1.0$ kcal/mol) [15•]. In contrast, only a few contact residues dominate the binding energetics of two other protein–protein complexes of known structure [16,17]. The general principles regarding the relationship between structural versus functional contacts await the availability of many more such detailed mutational analyses.

The molecular basis for the fine antigen specificity of an antibody fragment was investigated by determining the X-ray crystallographic structures of antibody complexes with two closely related steroid hormones [18•]. Fine specificity in this case is apparently mediated by rearrangement of ordered water molecules and an altered conformation of a single hydrogen bond. The recognition features of antibodies binding to protein antigens are different, however, and are probably more complex than those of antibodies binding to small molecules.

These studies bring us a step closer to an understanding of how the structural features of an antibody–antigen complex contribute to the affinity and specificity of the binding reaction.

Humanization

Antibody 'humanization' is widely used to address the problems of immunogenicity and inefficient secondary immune function that frequently beset the clinical usage of rodent monoclonal antibodies (reviewed in [19]). Humanized antibodies are commonly created by transplanting the antigen-binding loops, known as complementarity-determining regions (CDRs), from rodent to human antibodies. The additional recruitment of one or more framework region (FR) residue (non-CDR residue in the variable domains) from the parent rodent antibody is usually required in order to obtain efficient antigen binding. The identification of key rodent FR residues for incorporation can be laborious, particularly if their total number is minimized in order to reduce the potential risk of immunogenicity. FR optimization by phage display is an innovation that streamlined the humanization of an antibody against vascular endothelial growth factor (VEGF) [20•] and it is probably generally applicable.

Several insights have emerged from the structural comparison of murine and humanized versions of three different antibodies [21,22•,23]. Firstly, the murine CDR loop conformation is remarkably well preserved upon grafting on to a human FR, thus supporting the initial premise of humanization [24]. Secondly, the CDR loops are capable of modifying the conformation of the human FR so that it more closely resembles the murine parent antibody [21,22•]. This self-correcting mechanism has probably contributed in part to the successful humanization of, we estimate, at least 200 antibodies worldwide. Thirdly, relative movement of the light chain variable domain (V_L) with respect

to the heavy chain variable domain (V_H) is seen when comparing the human and murine antibodies. These quaternary structural changes may reflect the difference in affinity between murine and humanized antibodies [23].

Diabodies and triabodies

The construction of single-chain variable region fragment (scFv) molecules, with short linkers (5–10 residues) between the V_H and V_L domains, permits interchain but not intrachain pairing of the variable domains, resulting in the formation of a bivalent fragment known as a 'diabody' [25]. Bispecific diabodies are heterodimers of two 'crossover' scFv molecules in which the V_L and V_H domains of the two antibodies are present on different polypeptide chains [25]. Diabody fragments are one of the most promising routes for producing bispecific antibodies for clinical applications because they can be secreted from a single *E. coli* host and, in one case, have been directly recovered in yields approaching a gram per liter [26]. Moreover, the fraction of diabody preparation that is functional heterodimer, rather than inactive homodimer, has been improved by disulfide bond and 'knobs into holes' engineering [27]. Installing an interchain disulfide bond has also been found to enhance diabody stability *in vitro* and tumor localization *in vivo* [28].

For some therapeutic applications of diabodies, it is desirable to bestow them with effector functions and a long serum permanence time. These goals have been accomplished in a bispecific diabody in which one arm binds to serum IgG [29••]. Such diabodies support antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. Additionally, IgG binding increases the *in vivo* terminal half-life of the diabody by approximately sixfold, to 10 hours. A bispecific diabody in which one arm binds to the complement component, C1q, was shown to be effective in supporting complement-dependent cytotoxicity [30•].

scFv molecules in which the variable domains are connected without a linker may form noncovalent trimers known as triabodies. This was shown by gel filtration [31•,32], analytical ultracentrifugation [31•] and X-ray crystallography [33••]. All three antigen-binding sites are functional in at least some triabodies [31•,32]. The small size of triabodies (~72 kDa), in conjunction with their higher apparent affinity compared to scFv, make them attractive candidates for radio-immuno-imaging and radio-immunotherapy [31•].

X-ray crystallography of a triabody [33••] has revealed the anticipated trimeric structure [31•]. The triabody, with noncognate pairing of its V_H and V_L domains, was compared to a crystal structure in which the same V_H domain was paired with its cognate V_L domain [33••]. The conformation of the V_H CDR3 was found to be dependent upon the partner V_L domain. This is an important finding, as it represents a hitherto unknown source of structural and, presumably, functional diversity in the antigen-binding sites of antibodies.

Bispecific IgG

The advent of a robust route for producing bispecific human IgG (BsIgG) (M Merchant, P Carter, unpublished data; see Note added in proof) overcomes a major hurdle in the clinical exploitation of these molecules. The production of BsIgG by co-expressing two different antibodies is commonly very inefficient as a result of unwanted pairings between the component heavy and light chains [34]. These problems have been overcome, enabling the almost quantitative formation and efficient recovery of human BsIgG. The heavy chains were remodeled for heterodimerization using 'knobs into holes' mutations [35] and were optimized by phage display [36••] in conjunction with engineered disulfide bonds. Light chain mispairing was circumvented by using an identical light chain for each arm of the BsIgG. Antibodies with identical light chains but binding different antigens were identified from a very large scFv phage library [37] with a limited light chain diversity.

The tailoring of antibody pharmacokinetics

The therapeutic potential of antibodies is substantially broadened by the ability to customize their pharmacokinetic properties for particular clinical indications. Until recently, the major tool for modifying IgG pharmacokinetics involved the use of fragments to reduce their terminal serum half-life from up to 21 days down to as little as a few hours. In addition, the serum half-life of Fab′ antibody fragments has been increased by site-specific modification of their free hinge cysteine with polyethylene glycol [38].

Engineering the Fc regions of antibodies, which comprise C_H2 and C_H3 domains, is emerging as an alternative way of manipulating the pharmacokinetics of antibodies. The interaction between rodent IgG Fc and the neonatal Fc receptor, FcRn, has been mapped by X-ray crystallography and site-directed mutagenesis ([39] and references cited therein). Impairing the binding of IgG Fc to FcRn reduces the serum persistence of the mutated Fc fragment [39]. This strongly implicates FcRn in regulating IgG serum persistence and led to the prediction that the serum persistence of an Fc fragment might be extended by increasing its affinity for FcRn, whilst maintaining the pH dependence of binding [40••]. This prediction was borne out using Fc mutants that were identified by selection of phage display Fc libraries using FcRn [40••].

Fv redesign

scFv molecules that are disulfide-free and stable are very attractive for intracellular applications of antibodies — 'intrabodies' — since the intracellular redox environment may limit disulfide-bond formation and thereby compromise function. This represents a challenging engineering problem since an intradomain disulfide bond is an almost ubiquitous hallmark of the immunoglobulin fold that contributes to its stability [41]. A stable and functional scFv

lacking cysteines was constructed by DNA shuffling and phage display [42•]. This cysteine-free scFv, but not the corresponding disulfide-containing scFv, could be expressed in a functional form in the cytoplasm of *E. coli*. Evaluation of these mutations in the context of other scFv and the identification of alternative solutions will surely follow.

One of the boldest antibody engineering efforts of the past year was an attempt at the *de novo* design of the antigen-binding site of a Fab [43]. Unfortunately, the designed Fab did not bind the target antigen, cystatin. Differences between the model of the designed Fab and its crystal structure provide some clues as to how to refine the design. The complexity of this design problem may be increased by the cooperative influences on the binding energetics of residues that are remotely located from each other, as seen in other antibodies [44]. In addition, even with advances in molecular modeling, the conformation of the V_H CDR3 is difficult to predict from the corresponding sequence as it is highly dependent upon the local environment [45].

Cyclically permuted Fv molecules have been created in which the natural connectivity between β strands is permuted [46]. An immunotoxin that was constructed using the cyclically permuted Fv was found to have an affinity that approached that of the corresponding scFv fusion protein and also had comparable cytotoxic activity. The practical value of this interesting Fv redesign remains to be determined.

E. coli expression of antibody fragments

Secretion from *E. coli* is the most widely utilized method for the production of functional antibody fragments. This reflects the fact that *E. coli* expression is rapid and technically simple to perform. Moreover, phage technology has emerged as a rapid and robust way of identifying antibodies and it is readily coupled to *E. coli* expression of fragments without subcloning, thereby accelerating the antibody characterization process.

The Achilles heel of antibody fragment expression in *E. coli* is the titer variability between different antibodies. Expression titers are highly dependent upon the antibody primary sequence and culturing conditions and, usually to a lesser extent, the expression vector and *E. coli* host strain (reviewed in [7]). A basic tenet of biotechnology is the use of high-cell density fermentation to enhance the titers of proteins that can be expressed in *E. coli*. Indeed, fermentation technology has been used to obtain gram per liter titers of a humanized antibody Fab′ fragment [47]. Antibody fragment production by *E. coli* in fermentors is currently limited by the expense and availability of equipment, rather than the methodology, which has already been described in detail [7].

Several methods for enhancing the titers of functional antibody fragments have emerged from extensive probing of the complex inter-relationship between primary sequence and *in vivo* folding, stability, host-cell viability and expression (reviewed in [7]). A very useful and serendipitous observation was that the expression titer of antibody 4D5 and two other murine monoclonal antibodies was increased up to 10-fold by humanization [47]. Two other independent laboratories [48,49] have also enhanced antibody expression using this humanization strategy, leading us to believe that this approach will be widely used.

The recruitment of FR residues from a well-expressed humanized 4D5 fragment [47] into a poorly expressed murine fragment (McPC603) resulted in an up to 10-fold enhancement in the titer of the functional fragment. This improved expression apparently reflects an increase in the proportion of the protein that is soluble, as well as the improved viability of the host cells during induction [50]. Although successful, this elegant strategy seems unlikely to gain widespread use since it is rather labor intensive and has not, as yet, been demonstrated with additional antibodies.

Sequence diversification of antibody fragments, combined with phage display screening, is potentially one of the most far-reaching strategies for enhancing expression titers. A 10-fold increase in the apparent production levels of a scFv molecule was achieved with this approach, using mutator cells to create the sequence diversity [51•]. Other methods for sequence diversification (reviewed in [8,9]) can also be used for 'expression maturation'. For example, the expression titer and binding affinity of an anti-VEGF Fab were enhanced in parallel using CDR mutagenesis and phage display (H Lowman, personal communication). Randomization of the linker connecting the V_L and V_H domains in the scFv, in conjunction with phage display, resulted in an up to five-fold improvement in expression [52,53].

Constructing scFv molecules exposes several residues to the solvent (including hydrophobic residues) that are usually buried by association with constant domains in the context of IgG. Mutational analysis of the residues exposed by scFv construction leads to a 25-fold increase in the functional periplasmic expression of the 4-4-20 antibody [54]. This is a potentially generally useful strategy that could be combined with phage display selection in order to identify the optimal replacement residues.

In vitro transcription and translation of scFv

Large-scale mutational analyses of antibodies and, presumably, other proteins have been greatly facilitated by the development of a new method that bypasses both time-consuming cloning and *in vivo* expression steps [55••]. Briefly, a product of PCR mutagenesis is used directly in coupled *in vitro* transcription and translation. A similar amount of protein (~0.3 pmol) was apparently synthesized for all the scFv mutants, allowing their relative affinities for multiple related antigens to be determined by an enzyme-linked immunosorbent assay (ELISA) [55••]. The production of functional scFv molecules by *in vitro* transcription followed by translation has been optimized

by the addition of protein disulfide isomerase and chaperones, plus reduced and oxidized glutathione [56•]. The proportion of functional scFv was increased more than fivefold to ~50%, whereas the total amount of scFv produced was similar (8–10 pmol).

High-affinity antibody fragments from phage libraries

Antibody fragments with nanomolar [57] and even subnanomolar [37] antigen-binding affinities can sometimes be identified directly from very large (more than 10^{10} clones) naïve phage display libraries. In some cases, it is desirable to isolate antibodies that have a high affinity for antigens that also have a particular biological activity. For example, the library of Vaughan *et al.* [37] was used to obtain a panel of 21 scFv against the tyrosine kinase receptor MuSK, including four scFv that bind MuSK with high affinity (K_d < 10 nM) and activate the receptor [58••]. Similarly, agonist scFv antibodies have also been obtained against the human thrombopoietin receptor, cMpl (C Adams, personal communication) and leptin receptors (M Rodrigues, P Carter, unpublished data), suggesting that phage libraries may be a robust route for discovering agonist antibodies. We have panned the library of Vaughan *et al.* [37] in order to identify scFvs that bind to most (>85%) of the target antigens evaluated (P Carter *et al.*, unpublished data), albeit with typically lower binding affinities (K_d = 10–300 nM) than those reported for different targets [37,58••]. In our case, affinity maturation was not required for *in vitro* applications of the phage library-derived antibodies but will probably be necessary for some *in vivo* applications.

Affinity maturation by phage display involves the use of one or more low affinity clones in order to construct secondary phage libraries. This is followed by panning and screening in order to identify higher affinity clones (reviewed in [8,9]). The advent of robust affinity maturation methods has permitted the study of the relationship between antigen-binding affinity and target localization *in vivo*. Tumor targeting was undertaken using ^{125}I-labeled scFv molecules with varying affinities for the protein product of the *HER2/neu* proto-oncogene. The extent and specificity of tumor localization was significantly enhanced by increasing the antigen-binding affinity (K_d) from 320 to 1 nM [59••].

Tumor targeting of a scFv molecule against the neovasculature-associated antigen oncofetal fibronectin was greatly improved using both affinity-matured and also dimeric fragments [60••]. Excellent tumor localization of a diabody was observed by real-time IR photodetection. In some cases, minor femur localization was observed. This reflects the fact that small amounts of antigen are present in synovia and some cartilaginous structures, and that this anti-fibronectin antibody [60••], unlike the HER2 antibody [59••], cross-reacts with the corresponding murine antigen. Thus, this anti-fibronectin antibody is a promising tumor-targeting agent and is also an excellent experimental system for exploring the optimization of fragment affinity (and valency) simultaneously for tumor localization and tumor–nontumor specificity.

Selectively infective phage

A new phage display technology, known as 'selectively infective phage' (SIP), selects for binding events by directly coupling them to the restoration of the infectivity of a noninfectious phage (reviewed in [12,13]). In one of several current versions of SIP technology, a noninfectious phage is first created by replacing the two N-terminal domains of the phage M13 gene III protein (gIIIp), which are critical for infectivity, with a ligand-binding protein such as a scFv molecule. Infectivity is then restored upon the binding of the scFv phage to an antigen that is connected to the two N-terminal domains of gIIIp (Figure 1, arrow b). Phage propagation is thus strictly dependent upon scFv–antigen interaction. The power of SIP technology was demonstrated by the selection of scFv molecules with the best affinities and stabilities from a well-characterized model library [61,62•]. SIP technology circumvents the physical separation of binding and nonbinding clones of conventional phage displays, which is often inefficient. This has the potential to speed up the selection process and increase the enrichment of cognate over noncognate phages per cycle.

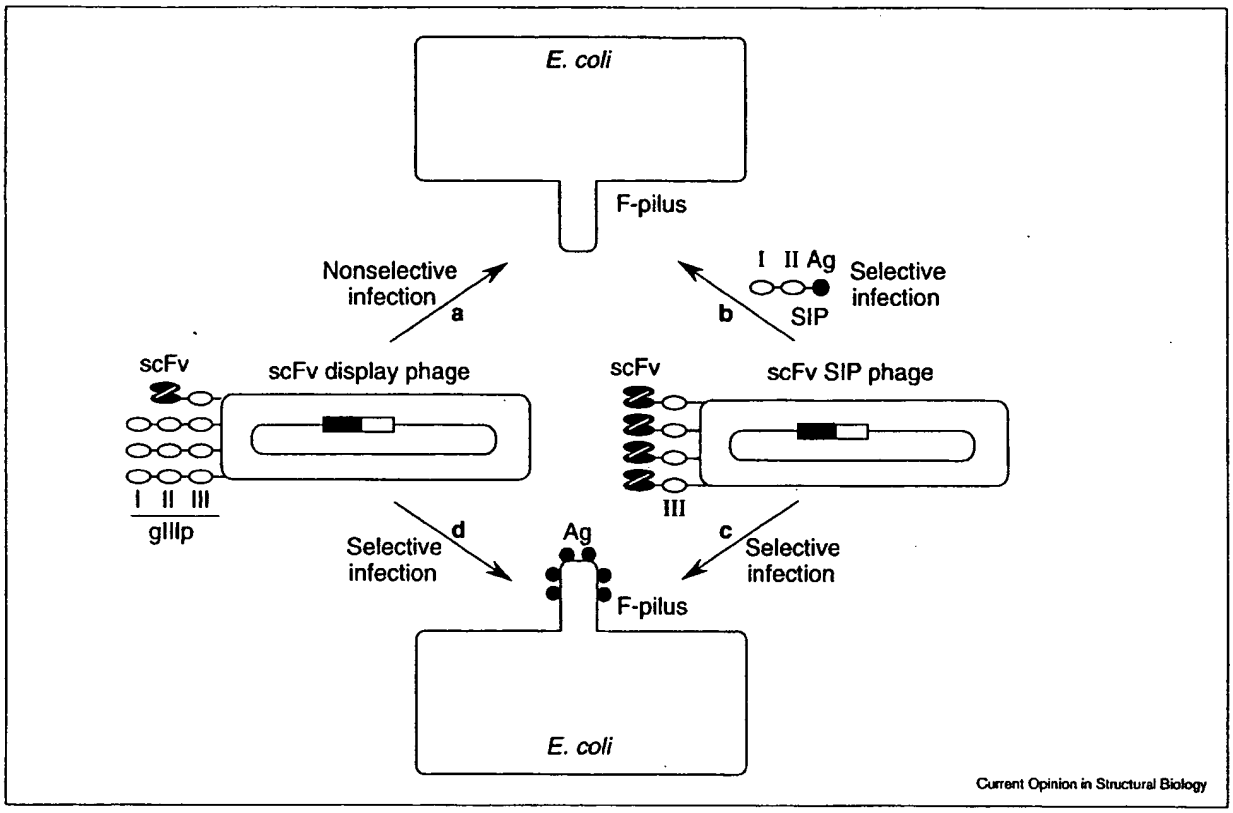
In another version of SIP technology, a peptide is genetically fused to the pilin component of *E. coli* F-pili. This abolishes infection by wild-type phages by preventing productive interaction with the F-pili [63•]. Infection can be restored by displaying on the phage a specific scFv that is capable of interacting with the peptide fused to the pilin (Figure 1, arrows c and d). A 2500-fold enrichment for the specific phage was obtained from a model library using a scFv against human cytomegalovirus [62•]. The extent to which SIP using pilin fusions is limited, specifically in terms of the peptide antigens that can be successfully displayed on the pilin, as well as the proportion of the bacteria displaying the antigen, remains to be determined.

SIP technology is well suited to co-selection of both interacting proteins, as suggested by a model system with gIIIp fusions [64]. The potential of SIP in selecting for catalytic antibodies is suggested by preliminary experiments in which an antibody that is capable of covalent catalysis was shown to restore the infectivity of noninfectious phage [65].

Ribosome display

The concept of ribosome display for linking phenotype and genotype was first demonstrated by Mattheakis *et al.* [66]. Briefly, a pool of DNA encoding 10^{12} random decapeptides was transcribed and translated *in vitro* and the nascent peptides on polysomes were selected by binding to an immobilized monoclonal antibody. The mRNA from the enriched pool of polysomes was then eluted, reverse transcribed into DNA and then PCR amplified in order to provide a substrate for the next selection cycle. After four cycles of this affinity selection, most clones

Figure 1



E. coli infection by scFv phage display and scFv SIP. The infection of *E. coli* by the scFv display phage is nonselective and is mediated by the interaction of the intact gene III protein (gIIIp) with the F-pilus on the *E. coli* host (arrow a). SIP phage are noninfectious because they carry the C-terminal domain of gIIIp (III) but lack the two N-terminal domains (I and II) that are required for infection. Infectivity can be restored by the interaction of the scFv with the corresponding antigen (Ag) that is either genetically fused or chemically coupled to gIIIp domains I and II (arrow b). Alternatively, infectivity can be restored by interaction of the scFv with the antigen displayed on the pilin component of the F-pilus (arrow c). Decoration of F-pili with peptides blocks the infectivity of the wild-type M13 phage [67••]. Therefore, scFv display phage can, in principle, be used for selective infection of cells expressing pilin–antigen fusions (arrow d). See [12,13] for additional details concerning SIP, including other variants of this technology.

encoded peptides that bound specifically to the antibody. Synthetic peptides corresponding to the clones bound to the antibody with affinities ranging from 7 to 140 nM.

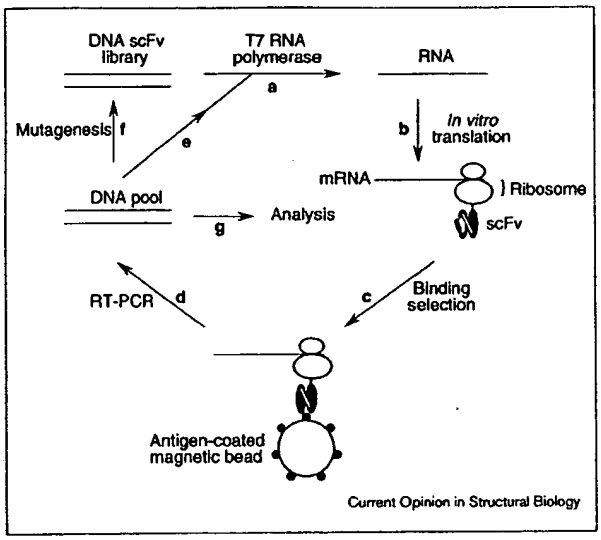
Ribosome display technology has recently been extended from peptides to antibody fragments [67••,68••] (Figure 2). The optimization of factors affecting mRNA stability and translation efficiency led to a 10^8-fold enrichment of cognate over noncognate scFv over five cycles of affinity selection [67••]. Even greater enrichments (10^4–10^5-fold per cycle) were realized by directly using the antibody fragment–ribosome–mRNA complexes as templates for PCR [68••].

Ribosome display has several potential advantages over phage display as a technology for identifying proteins or peptides that bind targets of interest, as well as modifying their affinity and substrate specificity. Firstly, larger libraries can be constructed with ribosome display compared to phage display. Secondly, ribosome display avoids the transformation and cloning steps that can be laborious with large phage display libraries and may result in the loss of diversity. Finally, additional genetic diversity can readily be introduced without cloning using ribosome but not phage display (Figure 2, arrow f). Optimized *in vitro* transcription and translation of scFv has led to up to 50% functional scFvs [56•]. It remains to be seen if this represents a significant limitation of SIP, although preliminary successes with this technology [67••,68••] augur well for its utility in antibody engineering.

Conclusions and future directions

One of the most important advances in antibody engineering of this past year is the refinement of ribosome display technology and its application to antibody fragments [67••,68••]. In ribosome display, an uncloned DNA library is constructed and then subjected to *in vitro* transcription and translation. The nascent peptides on polysomes are

Figure 2



A representative ribosome display cycle for the selection of scFv that bind to a specific antigen. A scFv library is transcribed with T7 RNA polymerase (arrow **a**) and then translated (arrow **b**) *in vitro*. The resulting mixture of scFv–ribosome–mRNA complexes is then selected for antigen binding (arrow **c**). The mRNA from the complexes is transcribed into DNA (arrow **d**) and used for the next round of selection (arrow **e**). Alternatively, this DNA pool can undergo mutagenesis to increase the sequence diversity (arrow **f**) prior to the next round of affinity selection. The DNA pool can also be used for analysis of the scFv, including cloning and sequencing, expression and characterization of antigen binding (arrow **g**). RT, reverse transcriptase.

then affinity selected by binding to an antigen. Ribosome display has the potential to supersede phage display, as it offers major possible advantages, including accessibility to larger libraries ($\geq 10^{12}$ clones). This is anticipated to lead to the identification of larger panels of clones against target antigens, including clones with higher antigen-binding affinities. In addition, ribosome display is particularly well suited to introducing additional genetic diversity without cloning. This will facilitate the manipulation of the affinity and antigen-binding specificity of antibody fragments.

In vitro transcription and translation of antibody fragments offers an exciting new alternative to *E. coli* expression of antibody fragments [55••,56•], as well as playing a central role in ribosome display. Moreover, this technology is key to a rapid method for the large-scale mutagenesis of antibodies that will doubtless find use with other proteins [55••].

Note added in proof

The paper referred to in the text as (M Merchant, P Carter, unpublished data) has now been accepted for publication [69••].

Acknowledgements

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- •• of outstanding interest

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